Express Mail No. EV336619448US

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Ralf M. Luche and Bo Wei
Application No.	:	09/847,519
Filed	:	May 1, 2001
For	:	DSP-14 DUAL-SPECIFICITY PHOSPHATASE

Examiner	:	Nashaat T. Nashed
Art Unit	:	1652
Docket No.	:	200125.422
Date	:	January 12, 2004

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF RALF M. LUCHE UNDER 37 C.F.R. § 1.132

Commissioner for Patents:

I, Ralf M. Luche, hereby declare that:

1. I am presently employed by CEPTYR, Inc. in Bothell, Washington, United States, the assignee of the above-identified application (hereinafter referred to as "instant application"), and I am a co-inventor of the subject invention described in the instant application.

2. The experiment described herein, performed at CEPTYR, Inc. with my knowledge, was performed according to the methods known in the art and described in the instant application. The results of the experiment demonstrate that the claimed polynucleotides encode a dual specificity phosphatase-14 (DSP-14) polypeptide that has the ability to dephosphorylate a suitable phosphorylated substrate.

3. **Preparation of wild type and substrate trapping mutant DSP-14 constructs.** Recombinant constructs encoding wild type DSP-14 polypeptide (SEQ ID NO:2) and DSP-14 substrate trapping mutant polypeptides were prepared according to methods known in the art and described in the specification (*see, e.g.,* page 7, line 12 through page 9, line 21; page 12, line 28 through page 13, line 18). A DSP-14 substrate trapping mutant retains the ability to bind a substrate, but displays a reduced ability to dephosphorylate a substrate when compared with the wild type DSP-14 polypeptide (*see* instant application, page 7, line 12 through page 8, line 5). One DSP-14 substrate trapping mutant, DSP-12 C147S, differed from wild type DSP-14 by having the cysteine residue at amino acid position 147 replaced with serine. A second DSP-14 substrate trapping mutant, DSP-14 D114A, was prepared that differed from wild type DSP-14 by having the aspartic acid residue at amino acid position 114 replaced with alanine. (*See id.*) The DSP-14 C147S and DSP-14 D114A mutant constructs were prepared by Retrogen (San Diego, CA) according to the vendor's protocol.

4. Vector pCMVTag2B (Stratagene, La Jolla, CA) was digested with restriction endonuclease BamHI (New England Biolabs, Beverly, MA) for 3 hours at 37 °C. The digested vector was incubated with Klenow polymerase (New England Biolabs) for 15 minutes at 25 °C to fill in the recessed 3' termini, and then incubated 30 minutes at 37 °C with calf intestinal phosphatase (New England Biolabs). The GATEWAY™ Reading Frame Cassette B (Invitrogen Life Technologies, Carlsbad, CA) was inserted into the pCMVTag2B vector by ligation with T4 DNA ligase (Invitrogen Life Technologies) overnight at 16 °C according to the supplier's instructions. LIBRARY EFFICIENCY® DB3.1™ competent *E. coli* cells were transformed with the ligated vector (GWpCMVTag2), and DNA was isolated by standard molecular biology methods.

5. DNA encoding the wild type DSP-14 polypeptide was amplified for insertion into cloning vectors using the following primers.

DSP-14 wild type antisense primer - (Dsp14 3' + attB2):

GGGGACCACTTTGTACAAGAAAGCTGGGTCTAGAGCTCCCTGCCATCCTCCTCCTCAC

DSP-14 wild type sense primer - (attB1 + Dsp14 5'):

GGGGACAAGTTTGTACAAAAAAGCAGGCTTAACATCTGGAGAAGTGAAGACAAGCCTCAA

GAATGC-

Vectors for expression of DSP-14 wild type (WT), DSP-14 C147S, and DSP-14 D114A were prepared as follows. The DSP-14 WT, DSP-14C147S, and DSP-14 D114A constructs were combined in a GATEWAY™ BP (Invitrogen Life Technologies) reaction with 1 µl of the DSP-14 constructs, 2 µl of the pDONR201™ vector, 11 µl TE buffer, 4 µl BP 5X reaction buffer (Invitrogen Life Technologies), and 4 µl BP Clonase™ enzyme (Invitrogen Life Technologies) for one hour at room temperature. After addition of Proteinase K (Invitrogen Life Technologies) to each reaction for 10 minutes, LIBRARY EFFICIENCY® DH5α™ *E. coli* cells were transformed with each entry vector construct.

6. FLAG® epitope-tagged DSP-14 WT, DSP-14 C147S, and DSP-14 D114A constructs were prepared by cloning the pDONR201™ DSP-14 WT and substrate trapping mutant constructs into the GWpCMVTag2 vector. The pDONR201™ constructs containing the DSP-14 WT, DSP-14 C147S, and DSP-14 D114A polynucleotides were linearized by digesting the constructs with Vsp I (Promega Corp., Madison, WI) at 37 °C for 2 hours. The DNA was purified using a QIAGEN PCR Purification kit (QIAGEN, Inc., Valencia, CA). Three microliters of the GWpCMVTag2 vector (100 ng/µl) were combined in a GATEWAY™ LR reaction with 6 µl linearized pDONR201™ DSP-14 WT, pDONR201™ DSP-14 C147S, or pDONR201™ DSP-14 D114A, 3 µl TE buffer, 4 µl Clonase™ Enzyme, and 4 µl LR reaction buffer (Invitrogen Life Technologies) for 1 hour at room temperature. After addition of Proteinase K (Invitrogen Life Technologies) to each reaction for 10 minutes, LIBRARY EFFICIENCY® DH5α™ cells were transformed with each expression construct. The DNA constructs were purified and the expression vectors or vector alone were transfected into 293-HEK cells (American Type Culture Collection, Manassas, VA) (maintained in DMEM, 10% (v/v) fetal bovine serum (FBS) at 37 °C and 5% CO_2), using the LIPOFECTAMINE™ 2000 reagent (Invitrogen Life Technologies) according to the manufacturer's instructions.

7. **Phosphatase Activity of DSP-14 and DSP-14 Substrate Trapping Mutants.** The phosphatase activity of DSP-14 WT was compared with the

activity of the substrate trapping mutants, DSP-14 C147S and DSP-14 D114A (*see, e.g.*, instant specification, page 18, line 21 through page 19, line 24; page 40, lines 15-26). DSP-14 WT and the substrate trapping mutant polypeptides expressed in 293-HEK cells were isolated by immunoprecipitation (IP). Untransfected cells and cells transfected with empty vector were included as controls. Twenty-four hours after transfection, the cells were harvested and lysed in IP buffer (25 mM Tris 7.2, 150 mM NaCl, 1mM EGTA, 1mM EDTA, 2mM DTT, 1% Triton, with Complete Protease Inhibitor (Roche Applied Bioscience, Indianapolis, IN)). The cell extracts (150 µl per sample) were combined with 10 µl anti-FLAG® M2 agarose beads (Sigma-Aldrich Co., St. Louis, MO) and incubated for 60 minutes at 4 °C. The agarose beads were separated from the cell extracts by centrifugation and then resuspended in 100 µl IP buffer. Two 25 µl aliquots of the resuspended beads were each incubated with 25 µl of substrate solution (1 µM 6,8-difluoro-4-methylumbelliferyl phosphate (DifMUP) (Molecular Probes, Inc., Eugene, OR) at room temperature for one hour. The agarose beads were then removed by centrifugation, and the supernatant was collected. Equal volumes (40 µl) of supernatant and water were combined to terminate the reaction. Catalytic dephosphorylation of substrate was determined by measuring the fluorescence intensity of the supernatant/water mixture at 400 nm and then subtracting from that measurement, the fluorescence intensity measured for the supernatants of the vector-only transfected control cells.

8. The DSP-14 WT and substrate trapping mutant polypeptide expression levels in cell extracts were analyzed by immunoblot. The agarose beads recovered from the DiFMUP activity assay described in paragraph 7 were washed with 1 ml TBS (25 mM Tris-HCl, pH 7.5, 150 mM NaCl). Duplicate samples of beads were combined. The beads were resuspended in 30 µl TBS containing 150 µg/ml 3X-FLAG® peptide (Sigma-Aldrich) to competitively release bound FLAG® fusion proteins, and then incubated with gentle rocking overnight at 4 °C. After incubation, the samples were centrifuged to pellet the beads, and 10 µl of each supernatant were combined with 10 µl of SDS-PAGE reducing sample buffer. The samples were heated at 100 °C for five

minutes and then applied to a 14% Tris-glycine SDS-PAGE gel (NOVEX®, Invitrogen Life Technologies). After electrophoresis, the separated proteins were electrophoretically transferred from the gel onto an Immobilon-P polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA). The PVDF membrane was blocked in 5% milk in TBST (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween-20), incubated with an anti-FLAG® M2 antibody (1:4000) (Sigma-Aldrich) for 1 hour at room temperature, washed 3 x 10 minutes with TBST, and then incubated with horseradish peroxidase (HRP) conjugated goat anti-mouse IgG (1:10,000) (Amersham Biosciences, Piscataway, NJ) for 30 minutes at room temperature. Binding was detected with the Western Lightning Chemiluminescent reagent, which was used according to the manufacturer's instructions (Perkin-Elmer Life Sciences, Boston, MA). Spot densitometry was used to determine the relative amount of cross-reactive FLAG®-DSP-14 WT and substrate trapping mutant polypeptides in each lane (ChemiImager 4400 software, Alpha Innotech Corp, San Leandro, CA). The relative catalytic activity in each cell extract was then determined by dividing the fluorescence intensity obtained using DiFMUP as substrate by the relative quantity of the corresponding FLAG®-DSP-14 polypeptide determined by spot densitometry.

9. As shown in the attached figure, expressed recombinant wild type DSP-14 polypeptide ("DSP14") catalytically dephosphorylated the phosphorylated substrate. The substrate trapping mutants of DSP-14, DSP-14 D114A (DSP14 DA) and DSP-14 C147S (DSP-14 CS), exhibited enzyme activity that was reduced by approximately 20% and 85%, respectively, relative to wild type DSP-14.

10. **U.S. Patent No. 6,268,135 (Acton)**: With respect to U.S. Patent No. 6,268,135 ('135) cited by the PTO as evidence that the claimed polynucleotides in the present application lack utility and are not enabled, I declare the following. I have reviewed the disclosure in '135, and as a highly skilled artisan in the molecular biology and protein tyrosine phosphatase (PTP) arts holding a doctorate in Biology since 1992, I respectfully conclude that the polynucleotide sequence set forth in SEQ ID NO:1 therein that encodes the polypeptide having the sequence set forth in SEQ ID NO:2, which the

patentees call cardiovascular system associated phospholipase (CSAPL), likely encodes an enzyme with phosphatase activity and not an enzyme with phospholipase activity. The PTP catalytic site motif -C-X_5-R-, can be found at positions 138-144 of SEQ ID NO:2 disclosed in the '135 patent. The conserved PTP catalytic site motif is well known in the PTP art and is present at amino acid positions 147-153 of DSP-14 (SEQ ID NO:2) of the instant application. Furthermore, the '135 patent teaches that the CSAPL polypeptide (SEQ ID NO:2, '135) is similar to dual specificity protein phosphatase 3 (Accession No. P51452, approximately 37% identical over amino acids 1-199 of CSAPL) (*see* '135, at column 56, lines 19-29). In concurrence with these teachings, I therefore conclude that CSAPL (SEQ ID NO:2) disclosed in '135 is a member of the dual specificity phosphatase family.

11. In contradiction to the teaching that CSAPL is a dual specificity phosphatase, the '135 patent also teaches that the CSAPL polypeptide disclosed therein (SEQ ID NO:2) possesses a phospholipase A_2 active site located at amino acid positions 131-138 of that sequence (column 8, lines 3-6). The '135 patent teaches that a phospholipase A_2 active site includes the consensus sequence CCX_2HX_2C ('135, at column 7, lines 60-65). I have inspected the sequence disclosed in SEQ ID NO:2 in '135 and find that amino acids 131-138, which are QGRVLVHC, fail to conform to the phospholipase A_2 active site consensus sequence (CCX_2HX_2C). I also find that no portion of SEQ ID NO:2 disclosed in '135 includes the phospholipase A_2 active site motif. I therefore respectfully submit that a skilled artisan would not reasonably conclude that a polypeptide having the amino acid sequence disclosed in SEQ ID NO:2 of '135 possesses phospholipase A_2 structure or activity, but would instead conclude that the polynucleotide encodes a member of the dual specificity phosphatase family.

12. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and that these statements were made with the knowledge that the making of willfully false statements and the like is punishable by fine or imprisonment, or both,

under Section 1001 of Title 18 of the United States Code, and may jeopardize the validity of any patent issuing from this patent application.

1-12-2004
Date

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Phosphatase Activity of DSP-14 WT and DSP-14 D114A and DSP-14 C147S Substrate Trapping Mutants